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RESEARCH ARTICLE

Antimicrobial and antiquorum-sensing activity of *Conocarpus lancifolius* Engl. (Combretaceae)

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ABSTRACT

Multidrug resistance to antimicrobial agents is a rapidly increasing problem. New strategies are required to reduce microbial resistance and pathogenicity. These include inhibiting microbial virulence factors, such as quorum-sensing (QS). In this study, the leaf and fruit extracts of *Conocarpus lancifolius* Engl. were investigated for their antimicrobial and QS inhibitory activities. The ethyl acetate extract of the leaf (EAL) recorded a remarkable inhibition of the Gram-negative bacteria; *Escherichia coli* and *Klebsiella pneumonia* (23 and 20 mm, respectively), using the agar well diffusion assay. Also, it showed the highest antifungal effect against *Candida albicans* (20 mm). Meanwhile, the ethyl acetate extract of the fruit (EAF) showed significant inhibition of the Gram-positive bacteria; *Staphylococcus aureus* and *Bacillus cereus* (20 and 21 mm, respectively). Measurement of the minimum inhibitory concentration (MIC) by the broth microdilution method indicated that Gram-positive bacteria were more susceptible to the antimicrobial effect of the investigated extracts. Particularly, EAL and EAF showed comparable activity to ampicillin against *B. cereus* (MIC, 312.5 µg/mL). The highest antifungal activity was recorded for the petroleum ether extract of the leaf (MIC, 625 µg/mL). Measurement of the inhibition diameter of pigment (PID) produced by the reporter strain, *Chromobacterium violaceum* indicated that EAL demonstrated higher QS inhibition (6 mm) compared to the standard, catechin (4.8 mm). In addition, the leaf and fruit extracts showed comparable activity to catechin (5 mm). The current study reported that *C. lancifolius* could be a promising antimicrobial and anti-quorum sensing drug candidate.

Keywords: *Conocarpus lancifolius*; Combretaceae; Antiquorum-Sensing Activity; Quorum-Sensing Inhibition; Antimicrobial Activity

INTRODUCTION

The invention of new antibiotics is challenged by the multi-drug resistance problem of many bacterial species (Hernandez-Rodriguez and Baquero 2022; Paczkowski et al. 2017). Therefore, there is a necessity to discover new therapeutic agents with new inhibitory mechanisms. Quorum sensing (QS) is described as a bacterial communication mechanism through which bacteria can sense their inoculum size, stimulate their growth, and enhances microbial pathogenicity (Hassan et al. 2016). QS mechanism is mediated by the production of signaling molecules (autoinducers, such as acyl-homoserine lactone) by bacterial strains, causing the transcription and high expression of certain virulence genes leading to biofilm formation (Fuqua and Greenberg 2002). QS inhibitors (QSIs) interfere with this mechanism and inhibit the subsequent expression of these virulence factors causing termination of the bacterial resistance (Hassan et al. 2016).

Natural products have been used in the control of several ailments. The antimicrobial properties of medicinal plants, either containing essential oils (such as *Pelargonium graveolens* and *Rosemary officinalis* L.) or other classes of bioactive molecules (such as *Allium sativum* and *Allium cepa*) have been reported by many researchers (Ghrairi et al. 2019; Kafa et al. 2022; Ruddock et al. 2005). *Conocarpus lancifolius* Engl. (Combretaceae), commonly known as " Damas", is an evergreen tree that can grow several meters in height. It is extensively cultivated in several countries of the Arabian Peninsula, including Saudi Arabia due to its ability to grow in extreme environments, including abiotic stresses e.g.,

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salinity, hot climate, and drought (Redha et al. 2011; Redha et al. 2012). Additionally, it tolerates biotic stresses as there are no reports of any plant pathogens or herbivores attacking this plant (Redha et al. 2011).

The phytochemical composition of *C. lancifolius* comprises simple phenolic acids (such as benzoic and cinnamic acids), tannins and polyphenols (such as catechin and quercetin), and phytosterols (such as β-sitosterol 3-*O*-glucoside) (Afifi et al. 2021; Al-Taweel et al. 2016). Several polyphenolic glycosides have been reported, including quercetin 3-*O*-β-glucuronide, kaempferol 3-*O*-rutinoside, 2,3,8-tri-*O*-methylellagic acid, 3-*O*-methyl ellagic acid 4-*O*-β-glucopyranoside, 3,3`,4`-trimethoxy 4-*O*-cyclopentanone ellagic acid (Al-Taweel et al. 2016; Saadullah 2015).

Diverse biological activities have been reported for *C. lancifolius*, including antidiabetic, inflammatory, antioxidant, cytotoxic, neuroprotective, and PPAR agonistic activities (Abdel Bar et al. 2022; Al-Taweel et al. 2016; Ayoub 2010; Mohammed et al. 2019; Saadullah et al. 2020; Saadullah et al. 2014). The antimicrobial activity of the aerial parts of *C. lancifolius* against several bacterial, fungal, and protozoal strains has been reported in the literature (Al-Musayeib et al. 2012; Ali et al. 2013; Javed et al. 2014; Mohammed et al. 2019). However, no previous studies addressed the anti-quorum sensing activity of this plant. Therefore, this work aimed to investigate the antimicrobial activity of the leaf and fruit extracts of *C. lancifolius* against different pathogenic microorganisms, including *Escherichia coli*, *Klebsiella pneumonia*, *Bacillus cereus*, *Staphylococcus aureus*, and *Candida albicans*. Also, the QS inhibitory activity of the different extracts of this plant against *Chromobacterium violaceum* was evaluated.

MATERIAL AND METHOD

General experimental procedures

For solvent evaporation, a Rotavapor R -215/Vacuum controller V-850 (BUCHI, SWITZERLAND) was used. Solvents used in the preparation of the extracts, including petroleum ether, ethyl acetate, and methanol were of reagent grade (Sigma-Aldrich Co., St. Louis, MO, USA). Luria-Bertani (LB) broth or agar (Sigma-Aldrich) was used in maintaining the bacterial cultures, whereas Sabouraud dextrose agar (SDA) was used for the fungal culture (Merck Co., Darmstadt, Germany). Optical densities (ODs) were assessed at 600 nm using a Biotek® microplate reader, ELx808™ (Biotek Inc., Winooski, VT). An orbital shaker incubator (Labtech, Korea) was used in growing the microbial cultures.

Plant material

The leaves and fruits of *Conocarpus lancifolius* Engl. were collected from a farm in Al-Kharj city, Saudi Arabia in February 2020. The identity of the plant was confirmed by Prof. Ibrahim Mashaly, Professor of Ecology and Botany Department- Faculty of Science- Mansoura University- Egypt. A voucher specimen with ID#16722 was reserved at the herbarium of the Department of Pharmacognosy- College of Pharmacy- Prince Sattam Bin Abdulaziz University- Al-Kharj- Saudi Arabia. The leaves and the fruits of *C. lancifolius* were separated from collected branches, artificially dried in a hot-air oven at 50°C, and powdered for further use in the preparation of extracts.

Preparation of the crude extract

The hot air-dried powdered leaves (850 g) and fruits (400 g) of *C. lancifolius* were extracted by maceration with cold distilled methanol (4 x 2 L for the leaves and 4 x 1 L for the fruits). The powdered plant materials (separately in each case) were immersed in methanol for 24 hours, then filtered, and this step was repeated five times to ensure complete extraction of the active constituents. The combined alcohol extracts (separately in each case) were concentrated using a rotatory evaporator (Büchi, Switzerland) at 45 °C. The obtained total methanol extracts were divided into two parts; the first part represented the total leaf extract (TL) or total fruit extract (TF); while the remaining part was suspended in distilled water and partitioned successively, with petroleum ether (PE) and ethyl acetate (EA) to obtain four other extracts/fractions; PE leaf extract (PEL, 18.0 g), PE fruit extract (PEF, 3.0 g), EA leaf extract (EAL, 9.28 g), and EA fruit extract (EAF, 5.38 g). The different extracts were evaporated to dryness under reduced pressure and kept for further investigation (Fig. 1).

Antimicrobial activity

Microorganisms

The antimicrobial activity was assessed against a panel of microorganisms, including the Gram-positive bacteria; *Staphylococcus aureus* (DSM-799) and *Bacillus cereus* (ATCC#11778), the Gram-negative bacteria; *Escherichia coli* (DSM-498) and *Klebsiella pneumonia*, (DSM-681), and the yeast-like pathogenic fungus; *Candida albicans* (ATCC#10231). In addition, the reporter strain, *Chromobacterium violaceum* (ATCC #12472) was used in the QS inhibition assay.

Antimicrobial assays

Agar well diffusion assay

The initial screening was performed using the agar well diffusion technique using Mueller Hinton Agar (MHA) medium for the antibacterial assay and Sabouraud Dextrose Agar (SDA) medium for the antifungal assay (Haikal et al. 2021). The microbial inoculum (50 µL) of 1×10^6 CFU/mL was added to melted agar, poured into 15 cm plates, and allowed to solidify. A cork borer was used to cut the wells (6 mm diameter). To prepare the sample

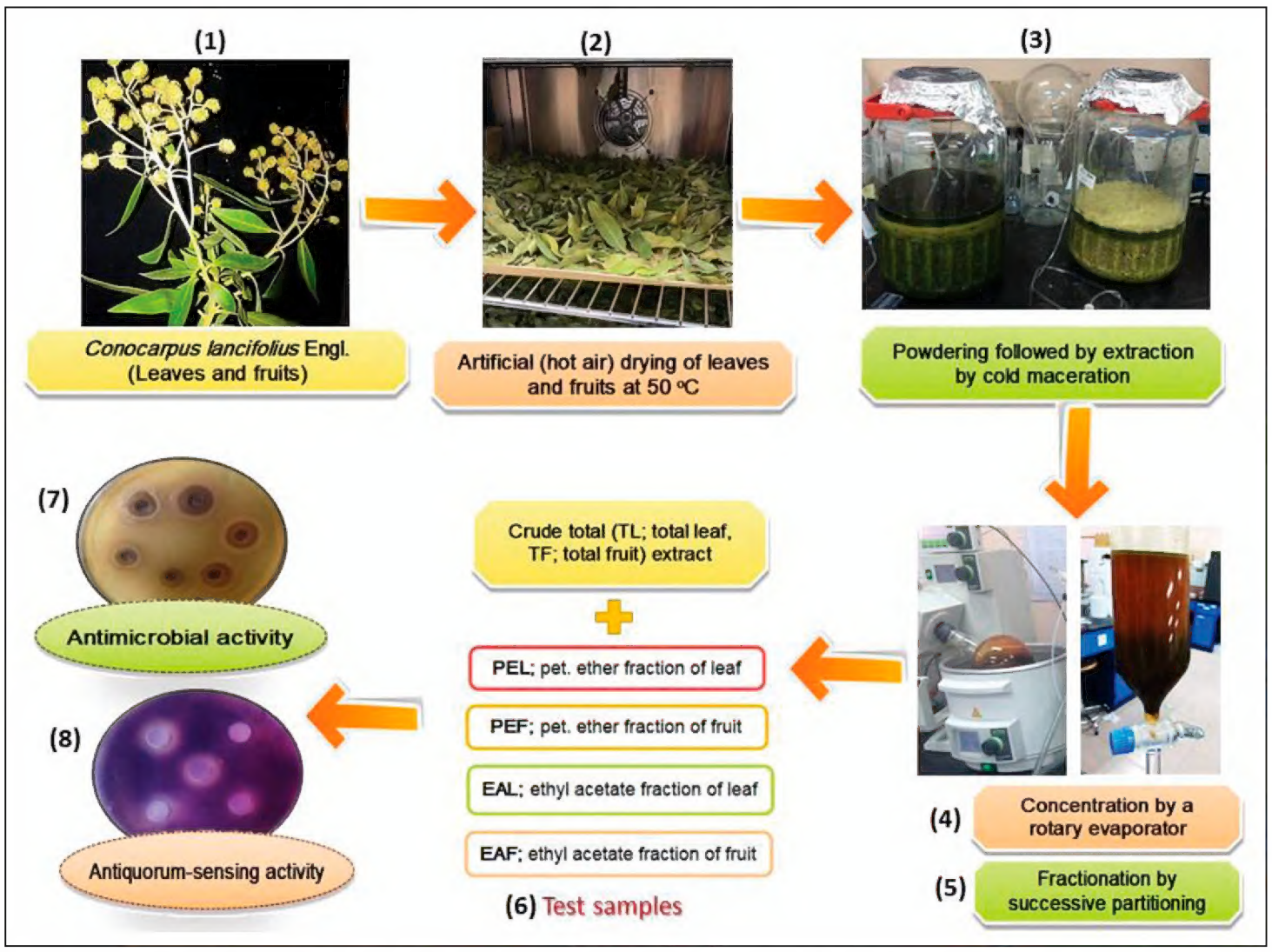
(1)
(2)
(3)
Conocarpus lancifolius Engl. (Leaves and fruits)
Artificial (hot air) drying of leaves and fruits at 50 °C
Powdering followed by extraction by cold maceration
(7)
Antimicrobial activity
(8)
Antiquorum-sensing activity
Crude total (TL; total leaf, TF; total fruit) extract
PEL; pet. ether fraction of leaf
PEF; pet. ether fraction of fruit
EAL; ethyl acetate fraction of leaf
EAF; ethyl acetate fraction of fruit
(6) Test samples
(4) Concentration by a rotary evaporator
(5) Fractionation by successive partitioning

Fig 1. A summary of the experimental procedure (steps 1-8) explaining the steps involved in the process of antimicrobial and antiquorum-sensing evaluation of the different extracts and fractions of *Conocarpus lancifolius* Engl.

solutions, the extracts (TL, TF, PEL, PEF, EAL, and EAF) were dissolved in DMSO at 10 mg/mL concentrations. Treatments were applied by adding 100 µL of the test sample solution per well. Then the plates were incubated at 37°C for 24 h. The diameters of the growth inhibition zone (IZDs) were determined in mm and the results were calculated after subtraction of DMSO activity. Ampicillin (69-52-3, Sigma-Aldrich, Saint Louis, MO, US) was used as a standard antibacterial compound whereas amphotericin B (1397-89-3, Sigma-Aldrich, Saint Louis, MO, US) was used as a standard antifungal drug.

Broth microdilution assay

The minimum inhibitory concentrations (MICs) were determined by the broth microdilution technique in 96-well plates (Abdel-Rahman et al. 2017). The overnight cultures of different bacterial strains were diluted to $OD_{600\,nm}$ of 0.01 (equivalent to 8 x10^6 cells/mL), while that of *C. albicans* was diluted to $OD_{600\,nm}$ of 0.5 (equivalent to 8 x10^7 cells/mL). The extracts, as well as standard antibiotics, were diluted by the two-fold serial dilution technique. Different dilutions of the extracts in DMSO (5000, 2500, 1250, 625, 312.5, 156.25, 78.125, 39.062, and 19.531 µg/mL) were applied to the diluted cultures in the microtiter 96-well plates as 5 % of the final volume in each well. The treated plates were incubated at 37°C for 24 h. The MIC values were determined as the lowest concentrations that completely inhibit the visible growth of the investigated microorganisms.

Antiquorum-sensing assay

The anti-pathogenic potential was checked by examining the QS inhibitory activity of the test extracts against *Chromobacterium violaceum* in an LB agar medium (Abdel-Rahman et al. 2017). *Ch. violaceum* culture was prepared by growing bacteria in LB broth followed by incubation at 28°C for 16-18 h in an orbital incubator running at 150 r.p.m. Cultures concentrations were then adjusted to 0.5 McFarland standard (equivalent to 1 x10^6 cell/mL). Next, *Ch. violaceum* culture (50 µL) was inoculated into LB agar medium (50 mL), poured into 15-cm plates, allowed to solidify, and afterward, wells were made using a cork borer. The different extracts solutions in DMSO (10 mg/mL), were then applied to the wells (50 µL/well). Catechin was used as a positive control at the same concentrations and volumes of the test samples, whereas DMSO was used as a negative control. Treated plates were incubated for 48 h at 30°C to check the inhibition of violacein pigment production around the treated wells. Inhibition of the bacterial growth resulted in a clear halo around the treated well, whereas quorum-sensing inhibition was demonstrated by the presence of a turbid halo harboring a non-pigmented area of the reporter strain, *Ch. violaceum*. Inhibition of bacterial growth (radius 1) by active extracts was measured as r1 in mm, while the inhibition of both growth and pigment production (radius 2) was measured as r2 in mm. The QS inhibitory activity was calculated by subtracting the radius of bacterial growth inhibition (r1)

Table 1: Antimicrobial activity (Inhibition Zone Diameters, IZD) of different extracts of C. lancifolius

Test samples[a]	Inhibition zone diameters (mm)[b]				
	Gram-negative bacteria		Gram-positive bacteria		Fungi
	E. coli	*K. pneumonia*	*S. aureus*	*B. cereus*	*C. albicans*
DMSO	--	--	--	--	--
Ampicillin	26	16	23	25	nt[c]
Amphotericin B	nt[c]	nt[c]	nt[c]	nt[c]	21
TL	19	17	22	20	16
PEL	--	--	--	--	--
EAL	23	20	18	21	20
TF	21	20	18	16	15
PEF	--	--	--	--	--
EAF	21	21	20	21	18

[a]Sample concentration: 10 mg/mL, Sample volume 100 μL/well. TL: total leaf extract, TF: total fruit extract, PEL: leaf extract, PEF: fruit extract, EAL: leaf extract, and EAF: fruit extract

[b]Results are calculated after subtraction of DMSO activity. *E. coli: Escherichia coli; K. pneumonia: Klebsiella pneumonia; B. cereus: Bacillus cereus; S. aureus: Staphylococcus aureus; C. albicans: Candida albicans*

[c]nt: not tested

Table 2: Antimicrobial activity; MIC (*μ*g/mL) of different extracts of *C. lancifolius*

Test samples	Minimum inhibitory concentrations, MIC (*μ*g/mL)*				
	Gram-negative bacteria		Gram-positive bacteria		Fungi
	E. coli	*K. pneumonia*	*S. aureus*	*B. cereus*	*C. albicans*
Ampicillin	78.125	312.5	39.0625	312.5	nt
Amphotericin B	nt	nt	nt	nt	0.61
TL	625	1250	625	312.5	1250
PEL	625	1250	2500	1250	625
EAL	1250	1250	312.5	625	1250
TF	1250	1250	312.5	312.5	1250
PEF	1250	1250	2500	1250	1250
EAF	625	1250	312.5	312.5	1250

* nt: not tested. TL: total leaf extract, TF: total fruit extract, PEL: leaf extract, PEF: fruit extract, EAL: leaf extract, and EAF: fruit extract

from the combined radius (r2) from the equation; QS inhibition = r2 – r1.

RESULTS AND DISCUSSION

The leaves and fruits of *C. lancifolius* were extracted with methanol to obtain the total methanol extracts (TL and TF) which were further fractionated using a nonpolar (petroleum ether, PE) and a medium polar solvent (ethyl acetate, EA) to divide the phytochemical components of the plant into two main groups of different chemical and biological properties. The content of the PE extract is mainly composed of terpenoids, phytosterols, lipids, and pigments. However, the EA extract mainly contains free/combined polyphenols, such as flavonoids, tannins, chlorogenic acid, benzoic, ellagic, and cinnamic acids (Abdel Bar et al. 2022; Saadullah 2015; Saadullah et al. 2020; Saadullah et al. 2016).

Antimicrobial activity

The antimicrobial activity of the different extracts of *C. lancifolius* was evaluated using the agar well diffusion method and the results are displayed in Table 1 as inhibition zone diameters, IZD (mm). Generally, the ethyl acetate fractions of both leaf and fruit extracts (EAL and EAF, respectively) showed the highest antibacterial and antifungal activities. The EAL extract showed the highest IZD against the Gram-negative bacteria; *E. coli* and *K. pneumonia* (23 and 20 mm, respectively), and the pathogenic yeast-like fungus; *C. albicans* (20 mm). Meanwhile, the EAF extract showed the highest IZD against Gram-positive bacteria, *S. aureus* and *B. cereus* (20 and 21 mm, respectively). Previous phytochemical research described the presence of several bioactive phenolic compounds in the ethyl acetate extract of *C. lancifolius* (Abdel Bar et al. 2022; Afifi et al. 2021; Saadullah et al. 2016). In addition to their recognized antioxidant effects, phenolic compounds are known for their significant antibacterial activity (Bouarab-Chibane et al. 2019). Thus, the obtained remarkable antibacterial activities of the ethyl acetate fractions can be attributed to the rich polyphenolic contents of the leaves and fruits of the investigated plant.

To measure the minimum inhibitory concentration (MIC, μg/mL) of the different extracts of *C. lancifolius*, the broth microdilution method was executed. The results showed that Gram-positive bacteria were more susceptible to the antimicrobial effect of most of the investigated

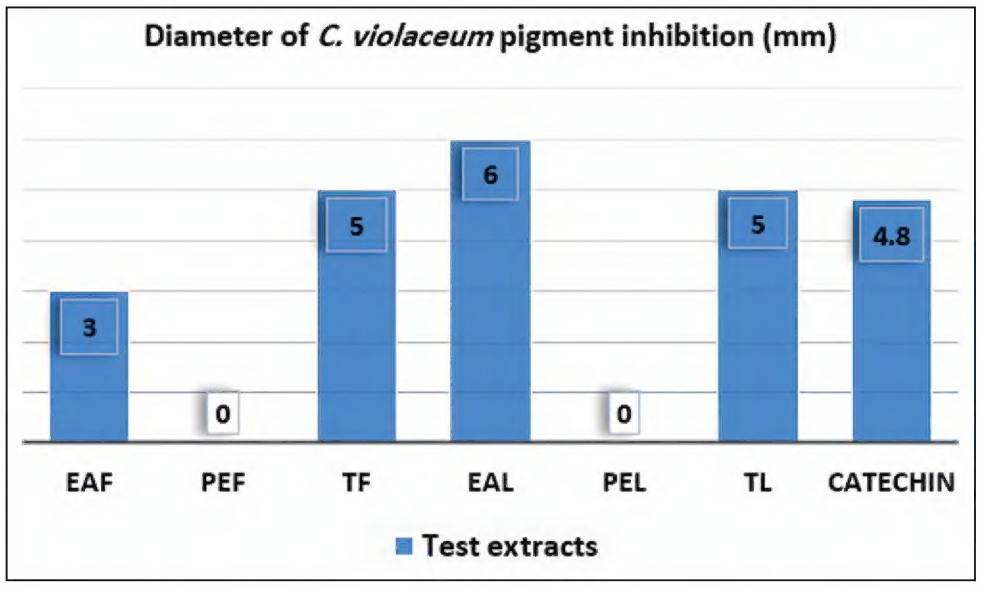

Fig 2. Antiquorum-sensing activity of the tested extracts recorded as diameters of *C. violaceum* pigment inhibition (mm). Results are calculated after the rebate of DMSO activity. TL: total leaf extract, TF: total fruit extract, PEL: leaf extract, PEF: fruit extract, EAL: leaf extract, and EAF: fruit extract.

plant extracts (Table 2). For *S. aureus*, EAL, TF, and EAF extracts showed the greatest antibacterial activity with a MIC of 312.5 µg/mL. On the other hand, TL, TF, and EAF extracts demonstrated comparable activity to the standard antibiotic, Ampicillin (MIC, 312.5 µg/mL). The greatest antifungal activity for the tested extracts was recorded for the PEL extract which showed the lowest MIC (625 µg/mL).

Antiquorum-sensing activity

The different leaf and fruit extracts of *C. lancifolius* were evaluated for QS inhibitory activity by the assessment of violacein pigment production of the reporter strain, *Ch. violaceum* (Hassan et al. 2016). Measurements of the pigment's inhibition diameter (PID) indicated that the EAL extract showed greater QS inhibition (PID, 6 mm) than the reference standard, catechin (PID, 4.8 mm). In addition, the TL and TF extracts showed comparable activity to catechin (5 mm). The EAF extract exhibited moderate QS inhibitory activity (PID, 3 mm). However, neither the petroleum ether fraction of the leaf nor that of the fruit showed no QS inhibition (Fig. 2).

Phytochemically, the ethyl acetate extract of the leaves of *C. lancifolius* (EAL) was shown to contain several flavonoids and phenolic compounds, including gallic acid, catechin, quercetin 3-*O*-β-glucuronide, kaempferol 3-*O*-rutinoside, dihydromyricetin, myricetin, syringetin 3-*O*-β-D-glucopyranoside, quercetin 3-*O*-β-D-glucoside, gallocatechin, and (-)-epigallocatechin-3-*O*-gallate (Abdel Bar et al. 2022; Afifi et al. 2021; Saadullah et al. 2016). Paczkowski et al. (2017) reported that flavonoids can inhibit biofilm formation and suppress virulence factors in *Pseudomonas aeruginosa* by inhibiting the QS mechanism through antagonizing the process of binding of the autoinducer to the QS receptors, LasR and RhlR. Therefore, the anti-QS activity of the EAL extract may be attributable to the high flavonoid content of this extract.

CONCLUSIONS

The leaf and fruit extracts of *Conocarpus lancifolius* Engl. (Combretaceae) were investigated for their antibacterial, antifungal, and QS inhibitory activities. The investigated plant extracts showed remarkable antibacterial activities against Gram-negative bacteria (*E. coli* and *K. pneumonia*) and Gram-positive bacteria (*S. aureus* and *B. cereus*) and showed antifungal activity against the pathogenic yeast, *C. albicans* based on the agar well diffusion assay. In addition, most of the plant extracts demonstrated great quorum-sensing inhibition (QSI). This study demonstrated that the remarkable antibacterial activities of the ethyl acetate fractions of the leaf and fruit extracts were attributed to their phenolic contents. Whereas the flavonoid content of these fractions was responsible for the observed QSI activity. This work suggested that *C. lancifolius* could be a promising antimicrobial and anti-quorum sensing drug candidate. However, future phytochemical studies are required to identify the bioactive components in the ethyl acetate fraction of both the leaf and fruit extracts of *C. lancifolius* accountable for these activities.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Authors' contributions

Fatma M. Abdel Bar designed the research. Ehssan Moglad, Soha El-Shaer, Menntallah M. Allam, and Hala A. Algahtani conducted the experiments. Fatma M. Abdel Bar analysed the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

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